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TITLE: Use of Adipose Derived Stem Cells to Treat Large Bone Defects

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14. ABSTRACT Trauma-induced injuries being sustained by our military men and women result in loss of multiple tissues, including associated vasculature and nerves. Current replacement technologies cannot address these kinds of injuries. Tissue engineering is still largely at the single cell stage and the tissues that are produced do not integrate well with surrounding tissues. Mesenchymal stem cells (MSCs) are an important tool but patients with large wounds may have reduced sources of stem cells and those stem cells that are present may be less robust as a consequence of trauma and medical treatment to suppress infection and inflammation. Our hypothesis is that effective repair of large defects requires the concerted processes of bone modeling and remodeling to create a functional marrow cavity, vascularization, and innervation and the best way to achieve this is by using autologous stem cells. Our objective is to develop technology to use adipose-derived MSCs to treat critical size segmental defects. Adipose-derived MSCs are attractive because of their relative abundance but there are still many issues that need to be resolved. We will: (1) develop methods for enriching the population of MSCs in adipose-derived cell preparations from rats; (2) determine if MSC-enriched adipose cells can be used to effectively treat large segmental defects using a rat segmental defect model developed in our group; and (3) optimize this technology for use in male and female animals. Adipose tissues from male and female normal Sprague Dawley rats and obese Zucker rats will be used as cell sources. MSCs will be enriched by selective removal of adipocytes and effectiveness will be determined using in vitro and in vivo assays. To test the ability of enriched MSCs to repair a critical size defect, cells will be loaded onto polymer composite scaffolds and implanted in a rat segmental defect (male in male; male in female; female in male; female in female) and healing assessed by microCT, histology, histomorphometry, immunohistochemistry, and biomechanical testing. This research will provide important new technology for treatment of bone injuries due to trauma. Most individuals have an adequate supply of fat tissue that can be used as a source of cells, but MSCs are in low abundance. Culture expansion dilutes MSCs with more committed cells. In contrast, our approach is to enrich the MSCs in the population by selective removal of more differentiated adipocytes.

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PROGRESS REPORT
Year 2 Progress

Aim 1: Enrichment of adipose derived stem cells (ADSCs) from rat fat pads.

We have been able to demonstrate that osteogenic ADSCs can be isolated in an enriched population in a reproducible manner by treating the heterogeneous population of rat fat cells with resveratrol. The results of these experiments will be presented at the 2009 International Association for Dental Research meeting. We were also able to show that MSCs from different anatomic sites (bone marrow, umbilical cord, subcutaneous fat) differed in their osteogenic potential. These results will be presented at the 2009 Orthopaedic Research Society.

Aim 2: Use of ADSCs to treat segmental defects.

We have established a protocol for ADSC isolation and osteogenic enrichment, enabling us to reproducibly test scaffold loading efficiency. These studies have provided information on optimal delivery. We have also completed characterization of our segmental defect model, including analysis of vascular ingrowth during defect healing. We anticipate initiation of our in vivo study within the next four months.

Aim 3: Male v. female ADSCs and response to treatment.

We have completed studies showing a difference in the osteogenic ADSCs present in subcutaneous fat as a function of age. To do this, we developed an algorithm for quantifying the MSC population in each fat sample. These results are being submitted to Stem Cells for publication and to the 2009 Tissue Engineering and Regenerative Medicine Society meeting. We will now expand our analytical methods to compare male and female populations in each age group. These experiments are expected to be complete by 7/1/09.

Summary

All projects are on track as described in the Statement of Work. Tasks outlined for Year 2 are complete unless noted above. These are anticipated to be complete by the end of 12 months of Year 2 funding.

Year 3 Statement of Work

Year 3 objectives and associated tasks remain as described in the original statement of work, attached below.

STATEMENT OF WORK

Specific Aim 1. To develop methods for enriching the population of MSCs in adipose-derived cell preparations from rats.

Year 1

Task 1: Establish the ratio of MSCs/adipocytes in inguinal fats pads and bone marrow in normal male Sprague-Dawley rats and obese male rats Zucker rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in male rats).

Task 2: Use assays above to test effectiveness of treatment with $1\alpha,25(OH)_2D_3$ (dose response/time course) for increasing the MSC/adipocyte ratio in cells preparations isolated from inguinal fat pads and bone marrow from male rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in male rats).

Year 2

Task 1: Test effectiveness of treatment with two additional apoptotic agents (dose response/time course) for increasing MSC/adipocyte ratio in cell preparations isolated from inguinal fats pads and bone marrow from male rats.

Year 3

Task 1: Prepare MSC-enriched adipocytes for use in segmental defects below.

Task 2: Prepare summary report.

Specific Aim 2. To determine if MSC-enriched adipose cells can be used to effectively treat large segmental defects.

Year 1

Task 1. Develop methods for loading $1\alpha,25(OH)_2D_3$ treated male MSC-enriched fat cells onto polymeric scaffolds.

Year 2

Task 1. Determine if treatment with alternate apoptotic agents modify loading efficiency.

Task 2. Determine if MSC-enriched adipose cells support segmental defect repair (in vivo microCT at 4 weeks; microCT at euthanasia; vascularity at euthanasia; histology and histomorphometric assessment of bone healing; immunohistochemistry for re-innervation; biomechanics).

Year 3

Task 1: Prepare summary report.

Specific Aim 3. To optimize MSC technology for use in male and female animals.

Year 1

Task 1: Establish the ratio of MSCs/adipocytes in inguinal fats pads and bone marrow in normal female Sprague-Dawley rats and obese female rats Zucker rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in female rats).

Task 2: Determine if the in vivo assay is sex-specific by testing enriched MSCs from male rats in female rats and enriched MSCs from female rats in male rats; compare bone, cartilage and fat formation by histomorphometry.

Year 2

Task 1: Use assays above to test effectiveness of apoptotic treatments (dose response/time course) for increasing the MSC/adipocyte ratio in cells preparations isolated from inguinal fat pads and bone marrow from female rats.

Task 2: Assess sex-specificity of apoptotic treatments by comparing female and male preparations in vivo for MSC/adipocyte ratio as described above.

Task 3: Assess effectiveness of female enriched MSCs in the segmental defect model using female rats.

Year 3

Task 1: Assess sex-specificity of enriched MSC effectiveness of by testing female enriched MSCs in the segmental defect model using male mice and male enriched MSCs in female rats.

Task 2: Prepare final report.

Osteoprogenitors are Enriched following Resveratrol-treatment of Adipose-derived Mesenchymal Cells

Erdman, Christopher P.[1,2]; Chen, Jiaxuan[1,2]; Olivares-Navarrete Rene[2]; Moyer, Hunter R.[2,3]; Williams, Joseph K.[3]; and Schwartz, Zvi[2]; Boyan, Barbara D.[2]

[1]The two authors contributed equally to this work

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Objective: Adipose tissue has been shown to contain a supply of mesenchymal stem cells (MSCs), however improved enrichment techniques are required before these cells can be used effectively. Resveratrol inhibits cyclooxygenase and activates Sirt1, which inhibit adipogenesis and induce apoptosis in adipocytes. This suggested that resveratrol could reduce the number of adipocyte progenitor cells and enrich the MSC population with osteogenic progenitors (OPCs). This study tested the hypothesis that resveratrol enriches MSCs and OPCs through an increase in cell number and percent of total population for both MSCs and OPCs. Methods: Adherent cells were isolated from the inguinal fat pads of Sprague-Dawley rats, plated at 5,000 cells/cm^2, and cultured in MSC growth media (GM) or osteogenic media (OM) (Lonza) containing 0, 12.5, or 25μM resveratrol for 7 or 14 days. Flow cytometry was used to assess expression of MSC (CD73+, CD271+, and CD45-) or osteoblast (osteocalcin) markers in the original population as well as following growth in GM or OM. Results: Resveratrol caused a dose and time-dependent increase in cell number and of the percentage of both MSCs and OPCs. In GM containing 25μM resveratrol, there was a 577-fold increase in MSCs at 7 days (5% of population), and a 106-fold increase in OPCs (21% of population) at 14 days. In OM, 25 μM resveratrol increased MSCs 27.8-fold (18% of population) at 7 days, and OPCs 29.9-fold (34% of population) at 14 days. Conclusion: These results show that MSCs and OPCs were present in the original adherent cell population and that resveratrol treatment enriched both cell populations. Resveratrol increased the cell number and the population percentage of MSCs and OPCs. Moreover, effectiveness of the treatment was reduced when the adherent cells were cultured in OM. Supported by Department of Defense; Children's Healthcare of Atlanta; NSF.

To be presented at the 2009 International Association for Dental Research

Reduced Surface Expression of CD44, CD49b, CD49e, and CD105 Associated with Growth of Human Bone Marrow Stem Cells Is Preserved During Growth in Osteogenic Differentiation Medium

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INTRODUCTION: Multipotent mesenchymal stem cells (MSCs) have been isolated from human bone marrow (BMSCs), and shown to have osteogenic and chondrogenic potential when cultured in defined differentiation media. Surface markers have been extensively characterized for the undifferentiated cells [1], but how expression of these markers changes during osteogenic and chondrogenic induction has not been described. It is clear that loss of the stem cell phenotype and acquisition of the bone or cartilage phenotype depend on the cell's ability to interact with its surrounding extracellular matrix [2,3]. For example, the alpha-2 subunit of the α2β1 integrin pair (CD49b) and the α5 subunit of α5β1 (CD49e) have been associated with osteoblast differentiation in a number of studies [4,5,11]. The objective of this study was to quantify changes in surface phenotypic expression during osteogenic and chondrogenic culture of human bone marrow-derived MSCs, focusing on those cell surface proteins that are specifically associated with cell/matrix interactions.

METHODS: MSCs were isolated from bone marrow aspirates from the iliac crest of normal adult donors [3] and expanded for 4 passages in growth media consisting of low glucose DMEM with 10% FBS. A portion of undifferentiated MSCs was resuspended in PBS to assess expression of surface markers by flow cytometry at day 0. Remaining cells were re-plated at 5000 cells/cm^2 with either growth media (GM) or osteogenic media (OM) consisting of GM supplemented with 1nM dexamethasone, 3mM beta-glycerophosphate, and 50 μg/μL ascorbic acid 2-phosphate. To assess effects of chondrogenic culture conditions, cells seeded in 1.2% Keltone alginate at a density of 12-15x10^{6}cells/ml were loaded on 24-well transwell insert membranes [6]. Once hydrogel discs were cross-linked in 100mM $CaCl_2$, samples were cultured in either incomplete chondrogenic medium (ICM) consisting of high glucose DMEM with 40μg/ml L-proline, 50 μg/mL ascorbate-2-phosphate, 0.1 μM dexamethasone, and 1% ITS+ or complete chondrogenic medium (CCM) consistent of ICM with 10ng/ml TGF-β3 and 50ng/ml BMP-6. After 7 days, cells in osteogenic culture were trypsinized and cells in chondrogenic culture were recovered by uncrosslinking the alginate with sodium citrate [7] and then incubated in 0.125% tyrpsin-EDTA solution with 2mg/ml collagenase at 37°C for 2 hours. Isolated cells were divided into aliquots of 1.25x10^{5}cells, washed in PermWash buffer, and incubated with PE-labeled anti-human mouse monoclonal antibodies specific for CD34, CD44, CD73, CD49b, CD49e, CD105 and CD146. Percent change in surface expression was calculated by normalizing the difference between day 7 and day 0 expression percentage to the day 0 expression percentage. Data were analyzed by ANOVA; * p< 0.01.

RESULTS: At T=0, BMSCs were positive for CD105, CD73, and negative for CD34. CD49b and CD146 expression was low whereas expression for CD44 and CD49e was high (Fig 1). The pattern of surface marker expression changed with time. Both control and osteogenic cultures exhibited down-regulation of CD146 (data not shown). However, cells cultured in OM exhibited distinct differences in surface marker expression compared to GM control cultures (Fig 2). CD49e and CD44 were upregulated in OM cultures but down-regulated in GM cultures. CD49b was down-regulated in both media conditions; however this effect was reduced in OM cultures, although this was not a consistent observation. CD105 was regulated in a similar manner to CD49b but the change in surface expression, while statistically significant, was less pronounced. BMSCs undergoing chondrogenic differentiation exhibited comparable changes in surface markers to cells undergoing osteogenic differentiation.

DISCUSSION: Surface marker expression varied as a function of both time and the medium in which the cells were cultured. CD146, which is a cell adhesion molecule generally used as a marker of endothelial cell lineage [8], decreased with time in culture but was independent of the medium used, suggesting that endothelial progenitor cells were present in low numbers and the GM media did not support endothelial lineage progression. BMSCs treated with OM in monolayer culture had significantly higher expression of the hyaluronan receptor (CD44), the α5 integrin subunit of the fibronectin receptor (CD49e), and the regulatory component of the TGF-β receptor (CD105) compared to GM cultures. The reduction in these surface markers observed in the cultures with time was mitigated by growth in OM or under chondrogenic culture. These results indicate that growth under appropriate conditions can help maintain or up-regulate cell adhesion molecules and surface proteins associated with tissue organization and cell differentiation [3,7,9,10]. Immunohistochemistry should be performed to confirm that the cell isolation process from tissue culture plates and hydrogels does not alter the surface phenotype. Gene expression of surface markers and proteins associated with osteoblasts and chondrocytes should also be investigated to determine if changes in the surface phenotype reflect post-translational regulation and to determine potential markers of differentiation. This study shows that exploring the temporal changes in surface phenotypic expression during osteogenesis and chondrogenesis can provide a better understanding of how cells interact with their surrounding environment throughout tissue development.

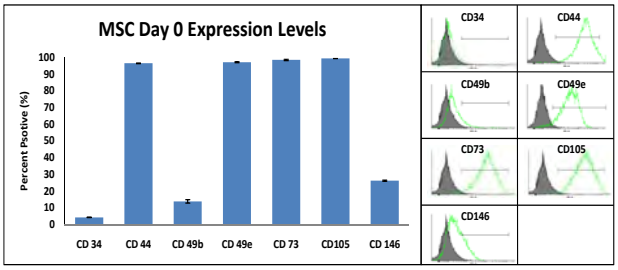

Figure 1

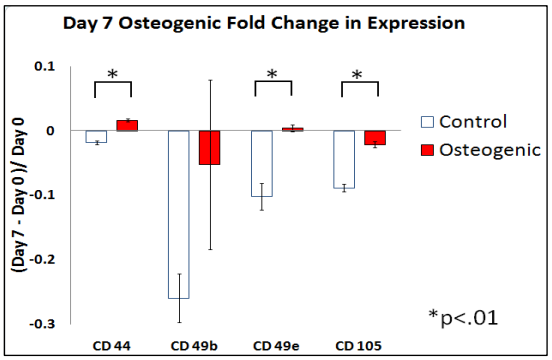

Figure 2

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INTRODUCTION

Trauma-induced injuries being sustained by our military men and women result in loss of multiple tissues, including associated vasculature and nerves. Current replacement technologies cannot address these kinds of injuries. Tissue engineering is still largely at the single cell stage and the tissues that are produced do not integrate well with surrounding tissues. Mesenchymal stem cells (MSCs) are an important tool but patients with large wounds may have reduced sources of stem cells and those stem cells that are present may be less robust as a consequence of trauma and medical treatment to suppress infection and inflammation. Our hypothesis is that effective repair of large defects requires the concerted processes of bone modeling and remodeling to create a functional marrow cavity, vascularization, and innervation and the best way to achieve this is by using autologous stem cells. Our objective is to develop technology to use adipose-derived MSCs to treat critical size segmental defects. Adipose-derived MSCs are attractive because of their relative abundance but there are still many issues that need to be resolved. We will: (1) develop methods for enriching the population of MSCs in adipose-derived cell preparations from rats; (2) determine if MSC-enriched adipose cells can be used to effectively treat large segmental defects using a rat segmental defect model developed in our group; and (3) optimize this technology for use in male and female animals. Adipose tissues from male and female normal Sprague Dawley rats and obese Zucker rats will be used as cell sources. MSCs will be enriched by selective removal of adipocytes and effectiveness will be determined using in vitro and in vivo assays. To test the ability of enriched MSCs to repair a critical size defect, cells will be loaded onto polymer composite scaffolds and implanted in a rat segmental defect (male in male; male in female; female in male; female in female) and healing assessed by microCT, histology, histomorphometry, immunohistochemistry, and biomechanical testing. This research will provide important new technology for treatment of bone injuries due to trauma. Most individuals have an adequate supply of fat tissue that can be used as a source of cells, but MSCs are in low abundance. Culture expansion dilutes MSCs with more committed cells. In contrast, our approach is to enrich the MSCs in the population by selective removal of more differentiated adipocytes

BODY

Aim 1: Enrichment of adipose derived stem cells (ASCs) from rat fat pads.

We have been able to demonstrate that osteogenic ASCs can be isolated in an enriched population in a reproducible manner by treating the heterogeneous population of rat fat cells with resveratrol. Resveratrol inhibits cyclooxygenase and activates Sirt1, which inhibit adipogenesis and induce apoptosis in adipocytes. This suggested that resveratrol could reduce the number of adipocyte progenitor cells and enrich the MSC population with osteogenic progenitors (OPCs). We tested the hypothesis that resveratrol enriches MSCs and OPCs through an increase in cell number and percent of total population for both MSCs and OPCs. To do this, adherent cells were isolated from the inguinal fat pads of Sprague-Dawley rats, plated at 5,000 cells/cm^2, and cultured in MSC growth media (GM) or osteogenic media (OM) (Lonza) containing 0, 12.5, or 25μM resveratrol for 7 or 14 days. Flow cytometry was used to assess expression of MSC (CD73+, CD271+, and CD45-) or osteoblast (osteocalcin) markers in the original population as well as following growth in GM or OM. Resveratrol caused a dose and time-dependent increase in cell number and of the percentage of both MSCs and OPCs. In GM containing 25μM resveratrol, there was a 577-fold increase in MSCs at 7 days (5% of population), and a 106-fold increase in OPCs (21% of population) at 14 days. In OM, 25 μM resveratrol increased MSCs 27.8-fold (18% of population) at 7 days, and OPCs 29.9-fold (34% of population) at 14 days. These results show that MSCs and OPCs were present in the original

adherent cell population and that resveratrol treatment enriched both cell populations. Resveratrol increased the cell number and the population percentage of MSCs and OPCs. Moreover, effectiveness of the treatment was reduced when the adherent cells were cultured in OM. We were also able to show that MSCs from different anatomic sites differed in their osteogenic potential. Moreover, the age and sex of the donor are important variables. The number of MSCs in rat adipose tissue decreases with age, but interestingly, the number of osteoprogenitor cells increases. However, the osteogenic potential of the adipose derived MSCs is not affected by donor age. The number of MSCs in rat adipose tissue was not sex-dependent, although there were sex-specific differences in the behavior of the cells under osteogenic media culture conditions.

Multipotent MSCs have been isolated from human bone marrow (BMSCs), and shown to have osteogenic and chondrogenic potential when cultured in defined differentiation media. Surface markers have been extensively characterized for the undifferentiated cells, but how expression of these markers changes during osteogenic and chondrogenic induction had not been described. It is clear that loss of the stem cell phenotype and acquisition of the bone or cartilage phenotype depend on the cell's ability to interact with its surrounding extracellular matrix. For example, the alpha-2 subunit of the $\alpha 2\beta 1$ integrin pair (CD49b) and the $\alpha 5$ subunit of $\alpha 5\beta 1$ (CD49e) have been associated with osteoblast differentiation in a number of studies. The objective of our study was to quantify changes in surface phenotypic expression during osteogenic and chondrogenic culture of human bone marrow-derived MSCs, focusing on those cell surface proteins that are specifically associated with cell/matrix interactions.

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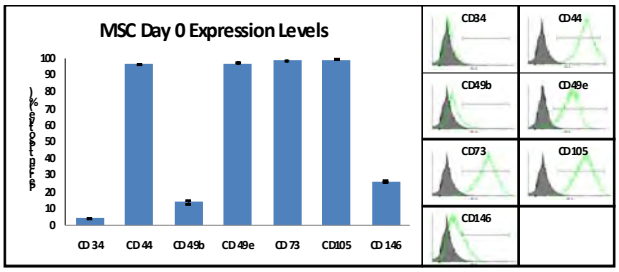

Figure 1

At T=0, BMSCs were positive for CD105, CD73, and negative for CD34. CD49b and CD146 expression was low whereas expression for CD44 and CD49e was high (Fig 1). The pattern of surface marker expression changed with time. Both control and osteogenic cultures exhibited down-regulation of CD146 (data not shown). However, cells cultured in OM exhibited

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Figure 2

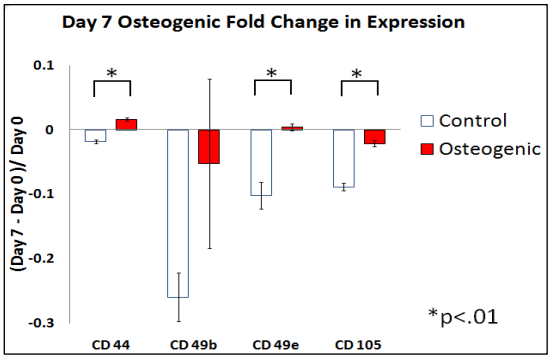
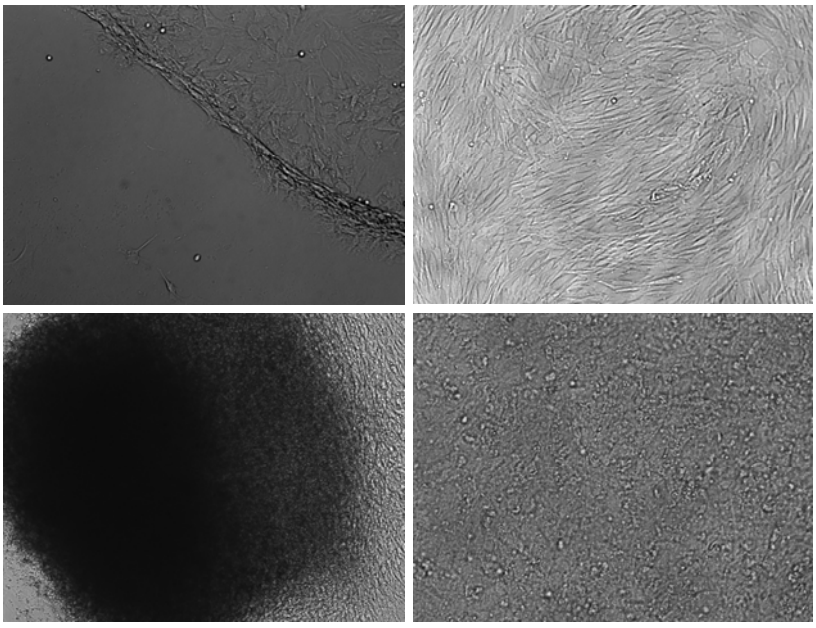


Figure 3



One of our goals was to determine if treatment with 1,25(OH)2D3 could be used as an alternative method for enriching osteogenic progenitor cells. The morphology of the ASCs obtained from rat inguinal fat pads was monitored in 2-D monolayer culture under various osteogenic differentiation media conditions (Fig 3). After 48 hours of culture, the cells demonstrated a clustering along the periphery of the well

that seemed to function as an anchor for the monolayer that was present in the center. By day 5, cells were fully confluent and the monolayers were treated with osteogenic media consisting of dexamethasone (Dex), 1,25 dihydroxy vitamin D3 (D3), or both Dex and D3 stimulating the cells down the osteogenic lineage. Dense nodules of cells were also observed in the monolayer as a product of cells piling on top of each other. After 15 days in osteogenic media, the monolayers exhibited areas of mineralization regardless of the osteogenic stimulus added.

Quantification of the mineralization of adipose derived stem cells from rat fat pads has also been conducted using a quantitative calcium assay that measures both intracellular and extracellular calcium (Fig 4). Cells were plated at a density of 5000 cells/cm^2 and cultured in either DMEM or alpha-MEM with the osteogenic stimuli detailed previously. Control media consisted of either DMEM or alpha-MEM with 16% fetal bovine serum (FBS), 3 mM beta-glycerophosphate (β-GP), 50 µg/mL ascorbic acid 2-phosphate, and 1% penicillin-streptomycin. Osteogenic media consisted of control media plus 1 nM Dex, 10 nM D3, or both 1 nM Dex and 10 nM D3. After 4 weeks of culture in osteogenic medium, cells cultured in alpha-MEM displayed similar values for total calcium, while cells cultured in D-MEM exhibited greater calcium values with osteogenic stimuli added to the media. Cells treated with both Dex and D3 and cultured in DMEM exhibited the greatest amount of calcium, eliciting this media condition as the optimal media to induce adipose derived stem cells down the osteogenic lineage.

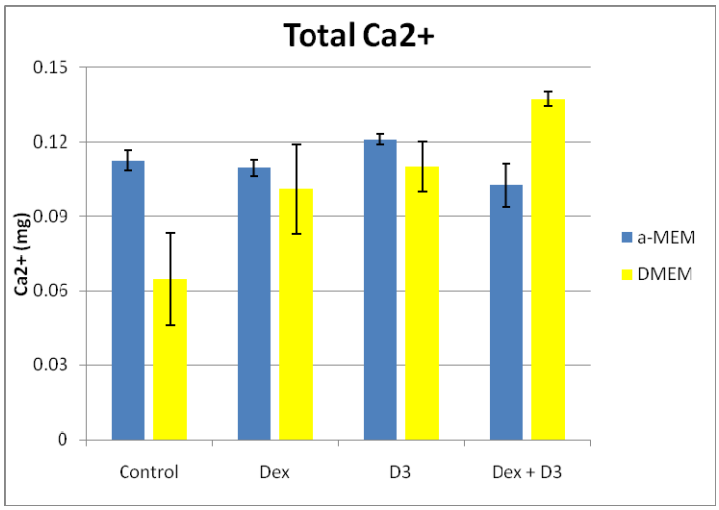


Figure 4

To test the capability of adipose derived stem cells obtained from rat fat pads to differentiate down the osteogenic lineage in 3-dimensional culture, poly-caprolactone (PCL) scaffolds were coated with a collagen gel and a bolus of cells were delivered to the scaffold/collagen construct. The same media conditions as the 2-D quantitative calcium assay discussed previously were used for the 3-D in vitro study. The scaffold height was 4.5 mm with 500,000 cells being delivered to the scaffold. Micro-CT scans were conducted at 5 and 8 weeks and a representative scan of the Dex and D3 group is displayed to the right at each respective time point. All groups displayed greater bone volumes at week 8 in comparison to week 5, displaying that the cells are capable of differntiating and mineralizing the PCL constructs (Fig 5). As with the quantative calcium assay, the media containing Dex and D3 displayed the greatest amount of mineralization, further purpoting this media condition as the optimal condition to drive adipsoe derived stem cells down the osteogenic lineage.

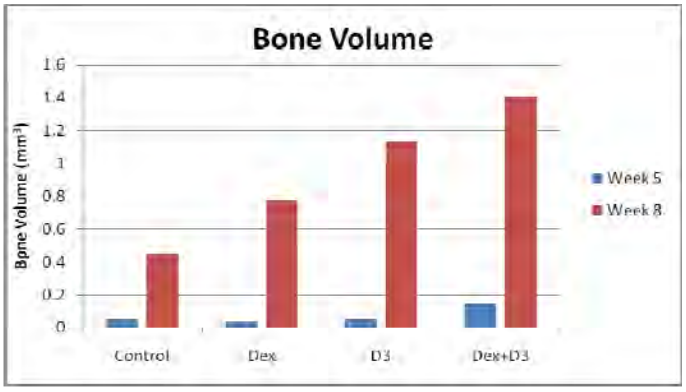


Figure 5

Week 5 Week 8

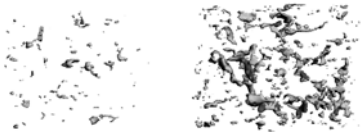
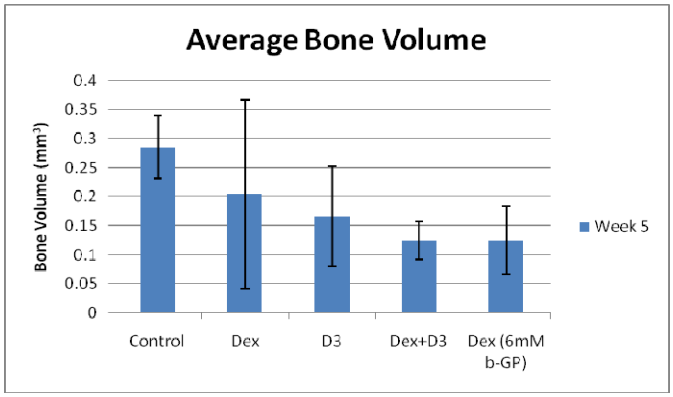
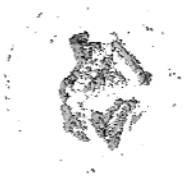


Figure 6



Top View

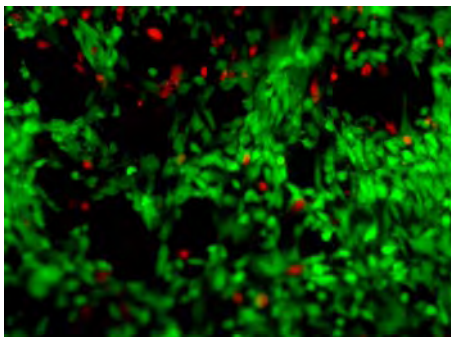


Side View

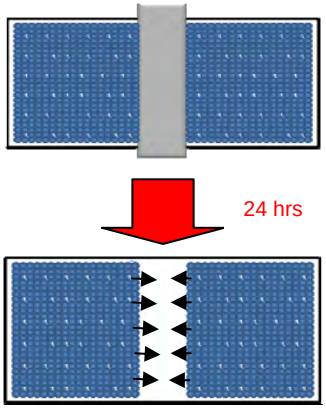
The potential of adipose derived stem cells obtained from human tissue have also been tested in 3-dimensional culture (Fig 6). For this study, 6 million cells were loaded onto 9 mm long PCL scaffolds. The same media conditions as the 2-D quantitative calcium assay discussed previously were used for the 3-D in vitro study, with a fifth group added containing a higher β-GP concentration of 6 mM. Micro-CT scans at week 5 demonstrate that the cells adhered to the PCL scaffold and further began to mineralize the scaffold (representative images above). Viewing the scaffolds from the top shows that a majority of the cells reside in the center of the scaffold, but some cells were found on the periphery and mineralizing the scaffold. Interestingly, the bone volume of the control group was the highest at week 5, but this is a relatively early time point and the osteogenic media may have more of an effect at later time points. Further studies will determine the optimal cell density to produce mineralization in 3-dimensional constructs using human adipose derived stem cells.

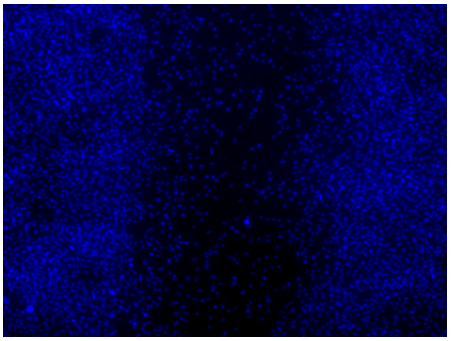
Recent 3-D studies have been conducted that have shown differing results from those previously outlined. In this study 3 million cells were seeded onto 4.5 mm long PCL scaffolds coated with lyophilized collagen, mimicking the design of the last study detailed. After seeding the scaffolds, cells were treated with a commercially available osteogenic media and growth media. Neither group showed mineralization after 5 weeks of treatment with osteogenic media. Further, there was an absence of sufficient cell matrix to support mineralization through the highly porous PCL scaffolds. To investigate this further, future work will look into the seeding efficiency and the ability of the cells to proliferate through the scaffold to provide a matrix by using Live/Dead stain to assess the distribution of cells through the scaffold during early time points (1 week) prior to receiving osteogenic stimulation (Fig 7).

Figure 7



An alternative cell delivery method for the repair of critically sized bone defects is being investigated with the use of nanofiber meshes. Nanofiber meshes offer the advantage of providing fibers on the same scale as extracellular matrix molecules and thus may present a hospitable delivery method for stem cells *in vivo*. Cell migration is currently being investigated with adipose derived stem cells. In these studies, cells are loaded at a high density of 40,000 cells/cm^2 onto an electro-spun PCL nano-fiber mesh (Fig 8). The high cell density helps to ensure that it is cell migration is the principal mechanism of bridging the gap rather than proliferation. 24 hours after seeding the barrier is removed and cell migration into the gap can be evaluated via DAPI nuclei stain under a fluorescent microscope. Processing of the images





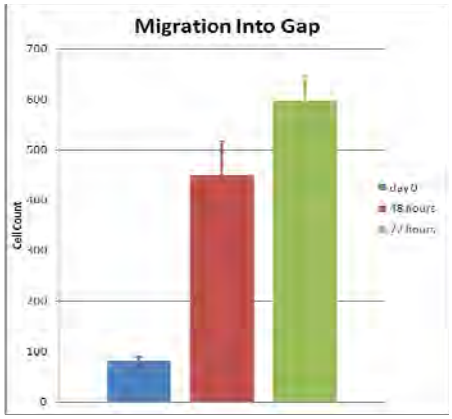


Figure 8

with Image J allows the number of cells in the gap to be quantified. Live/Dead stain confirmed adhesion and viable cells on the meshes. Early studies have sought to identify the proper time points to evaluate migration into the gap as well as the effect of collagen coating on the meshes to improve migration. Further, adipose derived stem cells seem to migrate and fill the gap at a faster rate than bone marrow mesenchymal stem cells. Future studies will investigate the differences in behavior between adipose derived stem cells and bone marrow mesenchymal stem cells on the meshes as well as assess the ability of adipose derived stem cells to undergo osteogenic differentiation on the meshes.

Aim 2. Use MSC-enriched adipose cells to effectively treat large segmental defects.

Our first set of experiments was to determine if resveratrol-enriched rat MSCs could be cultured on PCL scaffolds. For these experiments, 100x100x9 mm sheets of medical grade poly ε-caprolactone (PCL, Osteopore International, Singapore) with 85% porosity were cut with a 5 mm diameter biopsy punch to yield a cylindrical scaffold. The scaffolds were then briefly treated with 5M sodium hydroxide to roughen the surface and facilitate cell attachment. Scaffolds were then washed three times with sterile water and sterilized overnight via 70% ethanol evaporation. Sterile PCL scaffolds were then washed with excess sterile water three times and placed into a custom mold. Rat tail collagen type I (Trevigen, Maryland) was diluted with .05% acetic acid to 1.5 mg/mL, neutralized with 1M sodium bicarbonate, and aseptically pipetted into the mold to occlude the pores of the scaffold. The scaffold/collagen gel constructs were then placed in a -80°C freezer for 1 hour before being lyophilized overnight. Lyophilized scaffolds were then placed in a sterile scaffold holder and into 24 well low-attachment cell culture plates (Corning, New York) and stored until cell seeding.

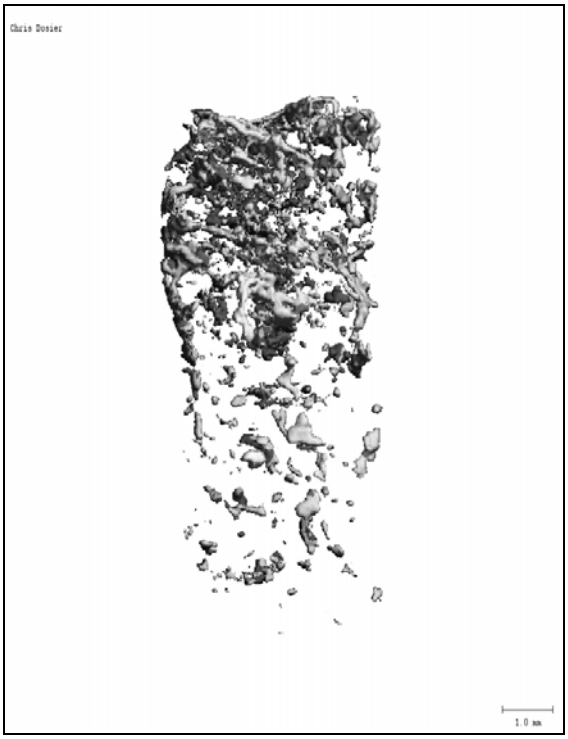
Cells treated with 25μM resveratrol for one week and untreated cells were trypsinized, counted, and reconstituted at a density of 3×10^4 cells/μL. 100μL (3×10^6) of cells were then carefully pipetted onto the tops of the scaffold/collagen constructs and allowed to attach to the surface. After 1 hour growth media (Lonza, Switzerland) were added to the culture well so that the cell-scaffold constructs were completely submerged in media. After 48 hours media was changed to osteogenic differentiation media consisting of α-MEM supplemented with 16% FBS, 1% penicillin-streptomycin, 50 μg/mL ascorbic acid 2-phosphate, 50 ng/mL thyroxine, 6 mM beta-glycerophosphate, and 1 nM dexamethasone. Media were changed twice weekly during cell culture. Cells were cultured dynamically on an orbital shaker at a rate of 6.5 RPM in a cell incubator. At 4 weeks mineralized matrix of the cell/scaffold constructs was determined through Micro-CT imaging using a VivaCT scanner (Scanco Medical, Switzerland).

Both resveratrol treated cells and untreated cells readily attached to the PCL-collagen constructs, producing extra cellular matrix which occluded the pores of the scaffold by 24 hours

(data not shown). After 4 weeks of dynamic cell culture in osteogenic media, micro-CT imaging showed that resveratrol treated and untreated cells both produced mineralized matrix throughout the entirety of the scaffold, with the resveratrol treated cells showing more of a qualitative distribution than untreated cells. Further, resveratrol treated cells produced significantly higher mineralized matrix with an average 33% more mineralized matrix than untreated cells at 4 weeks demonstrating that resveratrol treatment produces greater osteogenic differentiation than untreated adipose derived stem cells. Constructs cultured in media without osteogenic supplements showed little to no mineralization for both resveratrol treated and untreated cells (Fig 9).

Resveratrol Treated Cells Mineralized Matrix Volume (mm^3)	Untreated Cells Mineralized Matrix Volume (mm^3)
3.8523	3.524
4.0076	2.9353
4.6884	2.9867

Treated



Untreated

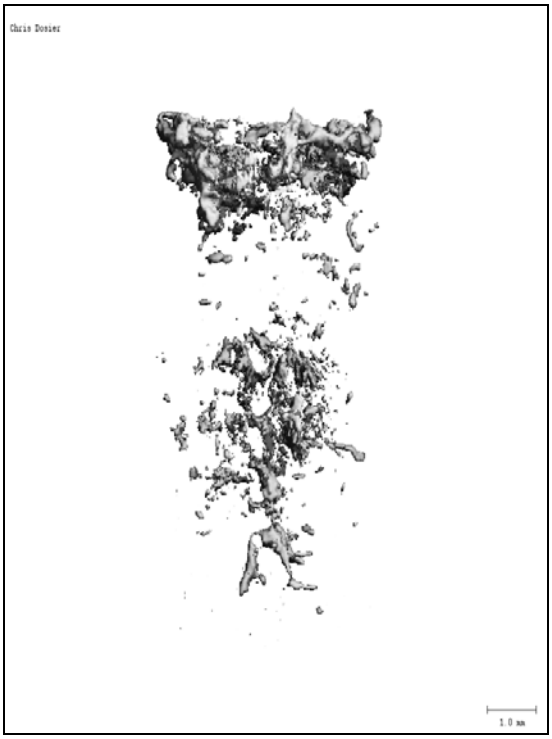


Figure 9

Based on these results, we initiated an in vivo study in which the cell/scaffold constructs were implanted in segmental critical size defects. The results from the implant study are now being assessed.

Aim 3. Optimize technology for use in male and female rats.

We did not pursue this aim as our funding was stopped at the end of year 2.

KEY RESEARCH ACCOMPLISHMENTS

- Multipotent stem cells can be isolated as an enriched population from rat subcutaneous fat pads.
- Enrichment can be achieved by treating the cells with resveratrol or with 1,25(OH)2D3, both of which are FDA-approved drugs.
- The number of MSCs present in fat and their osteogenic potential vary with age of the donor, with the number of MSCs decreasing with age and the number of osteoprogenitor cells increasing with age.
- Sex of the donor does not appear to affect the number of MSCs but it does affect their osteogenic potential and the number of osteoprogenitor cells present in the enriched population.
- Adipose derived stem cells can be loaded onto three dimensional scaffolds. Their ability to mineralize their extracellular matrix is increased when using populations enriched either with resveratrol or with 1,25(OH)2D3.
- Development of a critical size bone defect model in immunocompromised rat tibias.

REPORTABLE OUTCOMES

Presentations

Dosier CR, Kolambkar YM, Schwartz Z, Boyan BD, Guldberg RE. Cellular migration and osteogenic differentiation of adipose derived stem cells on electrospun nanofiber meshes. Selected for oral presentation at the 2010 ORS, New Orleans.

Erdman CP, Chen J, Olivares-Navarrete R, Moyer HR, Williams JK, Schwartz Z and Boyan BD. Osteoprogenitors are enriched following resveratrol-treatment of adipose derived mesenchymal cells. International Association for Dental Research, 2008.

Dosier CR, Lee CSD, Erdman C, Murphy M, Schwartz Z, Boyan BD, Guldberg RE, and Barry F. Reduced surface expression of CD44, CD49b, CD49e, and CD105 associated with growth of human bone marrow stem cells is preserved during growth in osteogenic differentiation medium. 2009 ORS.

Erdman CP, Olivares-Navarrete R, Williams JK, Schwartz Z, and Boyan BD. Pharmacological enrichment: a new approach to adipose-derived stem cell enrichment. 2010 Plastic Surgery Education Foundation Annual Meeting.

Lazin JJ, Erdman CP, Chen J, Olivares-Navarrete R, Moyer HR, Schwartz Z, and Boyan BD. Enrichment of adipose-derived mesenchymal stem cells. Society for Biomaterials, 2008.

Erdman CP, Chen J, Olivares-Navarrete R, Moyer HR, Williams JK, Schwartz Z, and Boyan BD. Enrichment of adipose-derived mesenchymal stem cells using resveratrol. 2008 Tissue Engineering and Regenerative Medicine International Society, European Division.

Dupont K, Garcia A, Sharma K, and Guldberg RE. Tracking human adult and fetal stem cells during segmental bone repair. 2009 ORS.

Kolambkar Y, Bajin M, Garcia A, Bellamkonda R, Hutmache D, Guldberg RE. Nanofiber structure and composition modulate human MSC migration and osteogenic differentiation. 2009 ORS.

Kolambkar Y, Dupont K, Mooney D, Hutmacher D, Guldberg RE. Effect of nanofiber mesh design on BMP-mediated segmental bone defect repair. 2009 ORS.

Boerckel J, Dupont K, Kolambkar Y, Lin A, Guldberg RE. In vivo model for assessing the role of load-bearing on tissue-engineered bone repair. 2009 ORS.

Publications

Guldberg RE. Spatiotemporal delivery strategies for promoting musculoskeletal tissue regeneration. J Bone Min Res 2009 24:1507-1511.

Boerckel JD, Dupont KM, Kolambkar YM, Lin AS, Guldberg RE. In vivo model for evaluating the effects of mechanical stimulation on tissue engineered bone repair. J Biomech Eng 2009 131:084502.

Piester A, Deutsch ER, Kolambkar Y, Hutmacher DW, Guldberg RE. Amniotic fluid stem cells produce robust mineral deposits on biodegradable scaffolds. Tissue Eng Part A 2009 15:3129-38.

Guldberg RE, Duvall CL, Peister A, Oest ME, Lin AS, Palmer AW, and Levenston ME. 3D imaging of tissue integration with porous biomaterials. Biomaterials 2008 29:3757-61.

CONCLUSION

This research program has resulted in the development of novel mesenchymal stem cell enrichment technologies that have the potential to permit autologous MSCs to be prepared from adipose tissue during surgical procedures to treat large bone defects. We have developed new three-dimensional in vitro assays that can be used to screen new technologies. Using these in vitro assays, we have shown that the enrichment of MSCs leads to better cell growth through three dimensional tissue engineering scaffolds and better osteogenic differentiation. Our results have also confirmed that the presence of MSCs in adipose tissue is reduced with age, although the number of cells committed to an osteogenic lineage is increased. What the consequences of this are to the development of regenerative medicine strategies is not yet known.